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Physalospora and Related Fungi

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Parasitism of *Calcarisporium parasiticum* on Species of *Physalospora* and Related Fungi

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THE fungi as parasites of economic plants have long been the objects of intensive study. The amount of time and effort given to the study of a causal fungus has been determined largely by the importance of the disease. Many contributions have been made to our knowledge of modes of parasitism, and the ways in which the parasites induce symptoms, but the fundamental questions of the physiological or chemical bases of parasitism, susceptibility and resistance remain largely unanswered. This is probably due to the priority given to research on practical problems and possibly in part due to the difficulties encountered in this type of physiological research.

Any study of parasitism involving the higher plants requires the growth of host plants in the field or in the greenhouse where conditions can be only partially controlled. Much time and space are required for growing the plants to the proper stage for inoculation and observation of disease development. Frequently only one experiment can be completed in a single season. As a result, the progress of studies of parasitism of higher plants is often slow. It is believed that the study of fungi parasitic on other fungi may yield important information on parasitism.

A number of types of parasitism have been recognized and parasites may be classified on the basis of mode of parasitism and effects on the host. The parasites may be internal or external, with or without haustoria, balanced (mostly obligate) or destructive. It is obvious that many different relationships may exist between host and parasite, and yet it is reasonable to believe that many of these follow the same general pattern. The obligate parasite is often considered as a type opposed to the non-obligate, because the parasite must obtain (so far as we know) all or an essential part of its nutrients from living cells. The non-obligate parasite would, therefore, differ from the obligate parasite in at least one factor.

Although this assumption is held by many people, its validity is open to question. Does the mere fact that a fungus can utilize the nutrients from a non-living natural or synthetic substratum alter the

method by which it obtains nutrients from living host cells? Perhaps a more workable approach to the question of differences among plant pathogens would lie in the study of the parasites that cause little or no obvious damage to the host during this period of their development, as opposed to parasites that kill the host or its parts by means of enzymes or toxins. These two groups of parasites have been designated as *balanced parasites* and *destructive parasites* (Bessey, 1935; Lilly and Barnett, 1951).

The balanced parasites of filamentous fungi include some of the most suitable organisms for the study of the basic principles of parasitism. The use of fungus hosts has many advantages: (1) Most of these fungi are rapid growers and complete their life cycle in a few days or weeks and it is possible to conduct a large number of experiments in a minimum time; (2) The hosts can be grown on synthetic media of known composition; (3) Comparatively little space and special equipment are required; (4) Rigid control of the environment is possible; (5) Effects of the parasite on the host are frequently reflected by an inhibition of growth which can be measured accurately.

Many host-parasite combinations are available to the interested research mycologist using modern methods and equipment. The discovery and publication of new facts and ideas on parasitism, even though they may seem unimportant when considered alone, is the only way we can hope to reach an understanding of parasitism as a way of life.

One of the earliest mycologists to recognize the parasitic habit of some species on other fungi was Brefeld (1872), who studied the growth of *Syncephalis* sp., *Chateocladium jonesii* and *Piptocephalis freseniana* on other Mucorales. He described and illustrated the haustoria of *P. freseniana* in the host mycelium. Van Tieghem (1875) demonstrated similar haustoria produced by other species of *Piptocephalis*. Matruchot (1900) was one of the first to determine the host range of a parasite fungus, using *P. tieghemiana*.

Recent studies of parasitism in species of *Piptocephalis* have included those by Dobbs and English (1954), Leadbeater and Mercer (1957), Berry and Barnett (1957), and Berry (1957). All species studied produce slender branched haustoria in the host cells and the host range is similar for all species, except *P. xenophila*. Dobbs and English found that this species is capable of parasitizing species of ascomycetes and imperfect fungi as well as several species of Mucorales. No species of *Piptocephalis* has been grown successfully in the absence of a living host.

Ayres (1933, 1935) found that *Dispira cornuta* is similar to known species of *Piptocephalis* in production of haustoria and in host range.

D. cornuta, however, differed in its ability to grow and sporulate on egg yolk, beef, swordfish and rat dung in the absence of a host fungus.

A second mode of parasitism among fungi was described by Burgeff (1924) for *Chaetocladium* and *Parasitella simplex*. These parasites made contact with and dissolved a hole in the wall of the host hypha, allowing the host nuclei to pass into a specialized basal cell of the parasite. This was followed by growth of the parasite. The genera *Piptocephalis*, *Syncephalis*, *Dispira*, *Chaetocladium* and *Parasitella* may be classed as balanced parasites, since they do not destroy the host mycelium and usually cause little or no visible damage to the host.

A third mode of parasitism is represented by *Papulospora stoveri* on *Rhizoctonia solani*, described by Warren (1948), and by *Rhizoctonia solani* principally on species of Mucorales and Peronosporales, described by Butler (1957). In this type the hyphae of the parasite twine around the host hyphae but form no haustoria or specialized points of contact. After a short period of growth the parasite kills the host and penetrates the cells. Numerous fungi over-grow and attack other fungi, but the degree of parasitism varies greatly in different species. The details of the manner in which these parasites kill the hosts are unknown, but it is assumed that the specific substances causing death of the host cells are of the nature of enzymes or toxins secreted by the parasite.

Perhaps similar to the destructive parasites are those fungi known to produce diffusable antibiotics and to inhibit the growth of other fungi at a distance. It is doubtful whether this type of action can be considered as true parasitism, but in some cases it is difficult to draw a sharp line between antibiotic action and parasitism.

So little is known about other parasites that their mode of parasitism cannot be classified. *Darlucia filum* on rusts and *Ciccinobolus cesati* on powdery mildews are among the few hyperparasites growing on hosts that are themselves obligate parasites of higher plants. The details of these relationships are little known. Other relationships awaiting detailed investigation are *Hypomyces* spp., *Verticillium* spp., and *Sepe-donium chrysospermum* on fruit bodies of fleshy fungi, *Gordyceps capitata* on *Elephomyces* and *Boletus parasiticus* on *Scleroderma*. Excellent general reviews of parasitism are given by DeVay (1956) and Yarwood (1956).

The purpose of this bulletin is to report the results of an extensive study of the parasitic relationship between *Calcarisporium parasiticum* Barnett and its fungus hosts. One of the main aims of this study was to learn more of the basic principles of the parasitism and resistance by the use of a parasite of other fungi.

DESCRIPTION AND CLASSIFICATION OF PARASITE

Calcarisporium parasiticum was first discovered in 1954, growing on a culture of *Physalospora glandicola* (Schw.) Stevens (*Dothiorella quercina* Cke. and Ellis) isolated from an oak tree suspected of having oak wilt. The white powdery appearance of the parasite made a striking contrast with the dark mycelium of the host (Fig. 1). Since the spores failed to germinate in water or on synthetic media and the fungus failed to develop further after germination on malt extract-yeast extract medium, the parasite was maintained on its host in tubes under refrigeration. Five additional isolates were obtained from cultures of *P. glandicola* in 1956. All isolates originated from different oak trees.

A search through the available literature failed to reveal any species to which this fungus could be assigned. However, it was undoubtedly closely related to two genera, *Calcarisporium* and *Hansfordia* (See Hughes, 1951). The well-developed conidiophores of the parasite sometimes bear whorls of 3 to 6 sporogenous cells (phialides) at 2 or more levels. The long verticillate conidiophores are believed to be more characteristic, although shorter conidiophores bearing fewer sporogenous cells are more abundant (Figs. 3, 4). Verticillate conidiophores are formed only under highly favorable conditions and may reflect a near-optimum nutritional relationship with the host.

It was concluded that this parasite was an undescribed species and for the sake of convenience in referring to the fungus in the present study it was necessary to give it a name. On the basis of genus description (Lindau in Engler and Prantl, 1900), it was believed that the species could be placed correctly in the genus *Calcarisporium*. Therefore, the parasite was described recently as a new species (Barnett, 1958) under the name of *Calcarisporium parasiticum*. Only a brief description of its outstanding characters, with illustrations, will be included in this paper.

Calcarisporium parasiticum, THE PARASITE

Mycelium hyaline, septate, sparsely branched, not extensive, mostly 1.5-3 μ wide; conidiophores hyaline, variable, simple or branched, short or long, reaching a length of about 0.5 mm, the main axis 3-4.5 μ wide; sporogenous cells (phialides) arising from the conidiophores singly, in pairs, or in whorls of 3-6 from different levels of the conidiophore, broader at the sterile basal portion, narrowed toward the fertile apical portion which continues to elongate, producing conidia in a loose ovoid or cylindrical cluster; conidia produced apically on successive new growing tips, hyaline, dry, narrowly obovate to elliptical, usually narrower at the base, 2.5-4 x 6-10 μ ; spore scars on short wart-like or peg-like projections. Parasitic on mycelium of certain other fungi. Known only from 6 isolates found growing on *Physalospora glandicola* in culture. A mutant

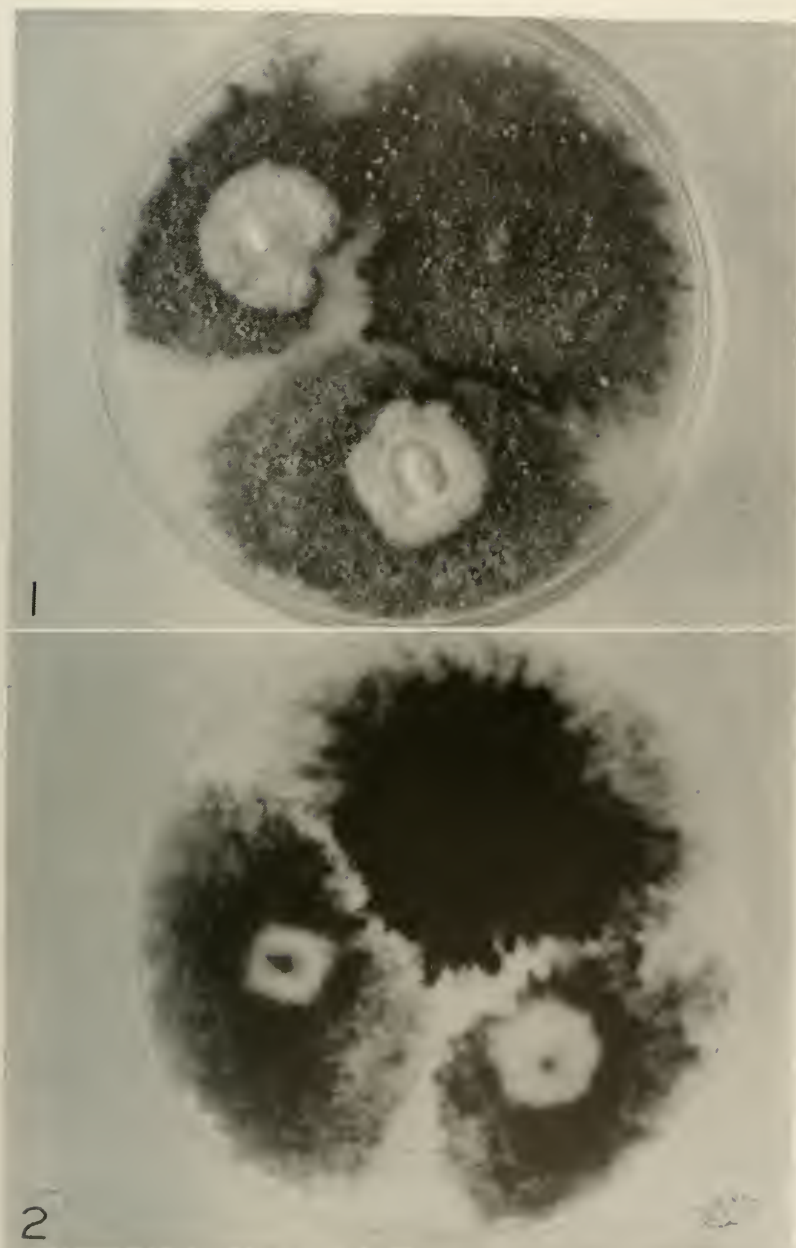


Figure 1, 2. *Calcarisporium parasiticum* growing on *Diptodia pinea*. (1) Top view of the culture showing the white mycelium of the parasite overgrowing the dark mycelium of the host. (2) The reverse side showing the area occupied by the mycelium of the parasite in which the host mycelium remained white.



Figures 3-12. *Calcarisporium parasiticum*. Conidiophores, conidia and stages of development until contact is made between host and parasite. (3) An unusually tall, well developed conidiophore showing the typical verticillate arrangement of the sporogenous cells. (4) A shorter conidiophore bearing fewer sporogenous cells, more common than the type shown in 3. (5) An immature conidiophore showing the typical formation of the lateral sporogenous cells after the terminal cell has produced spores. (6) A sporogenous cell bearing a loose cluster of conidia. (7) A sporogenous cell with conidia removed, showing the wart-like spore scars. (8) Conidia. (9) Germinating conidia showing production of secondary spores. (10-12) Time lapse drawings showing in sequence the positive tropic response of the host hyphae toward the germinated spore of the parasite, covering a period of three hours.

discovered during the course of this study produced a greater amount of aerial mycelium and fewer spores than the parent culture. Characteristics of the conidiophores, sporogenous cells, and conidia are shown in Figures 3-7.

Materials and Methods

The experiments performed in this study were so varied that most of the details are given with the description of the individual experiment. A few general methods that apply to most of the experiments are given here. Glucose-yeast extract agar was used as the standard medium for many experiments. It has the advantage of being easy to prepare, is an excellent medium for the growth of most fungi in Petri dishes, and is clear enough for direct microscopic study of the fungi during growth. The concentrations of the ingredients were modified as desired. Concentrations given throughout the paper are expressed in grams per liter. For example, a glucose-yeast extract (10-1) agar medium contains 10 g. of glucose, 1 g. of yeast extract, and 20 g. of agar per liter. Media not containing a natural product, such as yeast extract, were supplemented with KH_2PO_4 , 1 g.; MgSO_4 , 0.5 g.; and microelement solution,¹ 2 ml. The pH was adjusted to 6.0 before autoclaving at 15 pounds steam pressure for 15 minutes. Each Petri dish contained approximately 20 ml of medium. Liquid media were dispensed accurately, 25 ml per 250-ml Erlenmeyer flask. Unless otherwise stated, cultures were incubated in a constant temperature room at 25° C. and received 12 hours of artificial light each day.

Inoculations were usually made by bits of mycelium of host cultures on agar and a drop of spore suspension of the parasite. An ample supply of parasite spores for inoculum free from host cells was difficult to obtain in the early experiments. However, an abundance of spores was obtained after it was discovered how the parasite could be induced to sporulate on agar medium, in the absence of a host. This method is discussed in a later section of this bulletin. Host mycelium from liquid media was filtered off, dried overnight at 100° C. in a drying oven and weighed.

Extracts of mycelium of the host fungi were prepared by placing the mycelium of 5, seven- to ten-day-old cultures with 50 ml of distilled water in a blender jar and mincing the mycelium thoroughly. The

¹The microelement solution was prepared by dissolving $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, 724.5 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 439.8 mg.; and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 203.0 mg in distilled water acidified with sufficient sulfuric acid to yield a clear solution and made up to a volume of 1 liter. Each ml. of the above microelement solution contains 0.1 mg. each of iron and zinc and 0.05 mg. of manganese.

mycelial fragments were then removed by filtering through several thicknesses of filter paper and the filtrate was then sterilized by autoclaving or passing through a sintered glass filter.

Most of the test hosts were obtained from the West Virginia University Fungi Culture Collection. A few were obtained from the Centraalbureau voor Schimmelcultures at Baarn, and two from the American Type Culture Collection. Some were kindly furnished by Dr. C. T. Rogerson, of Kansas State College.

Experimental Results

HOST RANGE

During the course of this study approximately 40 species of imperfect fungi and ascomycetes were tested as possible hosts of *C. parasiticum*. This was done by placing bits of mycelium or spores of the test fungus on an agar plate and adding spores of the parasite. Mostly a malt extract-yeast extract (10-1) agar medium was used.

The appearance of typical conidiophores of the parasite on the host mycelium was used as the criterion of parasitism. Usually these could be seen and recognized in mass with the unaided eye, but cultures were always examined with the stereoscope or compound microscope. Conidiophores of the parasite were usually apparent after 2 or 3 days, but a period of 2 weeks was allowed for possible delayed development on some of the test fungi.

TABLE I. ORIGIN OF ISOLATES OF THE HOST FUNGI AND RELATIVE SUSCEPTIBILITY

HOST FUNGUS	NO. OF ISOLATES	SOURCE	STATE	SUSCEPTIBILITY
<i>Physalospora obtusa</i>	23	apple	W.Va.	high
<i>P. obtusa</i>	3	oak	W.Va.	high
<i>P. obtusa</i>	2	red bud	W.Va.	high
<i>P. obtusa</i>	1	honey locust	W.Va.	high
<i>P. obtusa</i>	1	quince	W.Va.	high
<i>P. glandicola</i>	20	oak	W.Va.	high
<i>P. glandicola</i>	2	oak	Kansas	high
<i>P. ilicis</i>	1	holly	Unknown	low
<i>Guignardia bidwellii</i> ..	1	grape	W.Va.	high
<i>Botryosphaeria ribis</i> ..	9	apple	W.Va.	high
<i>B. ribis</i>	2	sweet gum	W.Va.	high
<i>B. ribis</i>	1	red bud	W.Va.	high
<i>B. ribis</i>	1	oak	W.Va.	high
<i>B. ribis</i>	1	elm	Kansas	high
<i>B. ribis</i>	1	<i>Lonicera</i>	Kansas	high
<i>Diplodia pinea</i>	1	Scotch pine	Kansas	high
<i>D. pinea</i>	1	Scotch pine	W.Va.	high
<i>Coniothyrium</i> sp.	1	oak	W.Va.	very low
<i>Dothiorella</i> sp.	1	<i>Syringa</i>	Kansas	high

Results of the host survey showed that only six identified species and two isolates identified only to genus were hosts of *C. parasiticum*. These were as follows: *Physalospora obtusa* (Schw.) Cke. (*Spaeropsis malorum* Peck.), *Physalospora glandicola* (Schw.) Stevens (*Dothiorella quercina* Cke. and Ellis), *Physalospora ilicis* (Schl. ex Fries) Sacc. (*Phyllosticta ilicicola* (Cke. and Ellis) Ell. and Ev.) *Botryosphaeria ribis* G. and D., *Guignardia bidwellii* (Ellis) Viala and Ravaz, *Diplodia pinea* (Desm.) Kickx, *Coniothyrium* sp. and *Dothiorella* sp.

Numerous other species of fungi were tested but none supported growth of *C. parasiticum*. Spores of the parasite germinated in the presence of several of the species and contact was even established with some but there was no further development of the parasite. A list of the non-host species is given below.

<i>Alternaria tenuis</i>	<i>Gliocladium roseum</i>
<i>Ascochyta imperfecti</i>	<i>Glomerella cingulata</i>
<i>Ascochyta sorghi</i>	<i>Graphium rigidum</i>
<i>Bispora punctata</i>	<i>Hypoxyylon punctulatum</i>
<i>Ceratocystis fagacearum</i>	<i>Hypoxyylon tinctor</i>
<i>Ceratocystis fimbriata</i>	<i>Melanconium fuliginum</i>
<i>Chaetomium globosum</i>	<i>Pestalotia</i> sp.
<i>Chalaropsis</i> sp.	<i>Petriella</i> sp.
<i>Cladosporium</i> sp.	<i>Physalospora fusca</i>
<i>Colletotrichum phomoides</i>	<i>Physalospora miyabeana</i>
<i>Coniothyrium</i> spp.	<i>Physalospora mutila</i>
<i>Coryneum kunzei</i>	<i>Physalospora rhodina</i> (5 isolates)
<i>Curvularia lunata</i>	<i>Physalospora tucumanensis</i>
<i>Cytospora</i> sp.	<i>Physalospora zeicola</i>
<i>Dendryphiopsis atra</i>	<i>Pseudoplea trifolii</i>
<i>Diplodia zeae</i>	<i>Pyrenochaete</i> sp.
<i>Diplodia</i> spp.	<i>Rhizoctonia solani</i>
<i>Dothiorella</i> (<i>Cephalosporium</i>) <i>ulmi</i>	<i>Sclerotium rolfsii</i>
<i>Endothia parasitica</i>	<i>Sordaria fimicola</i>
<i>Fusarium culmorum</i>	<i>Stemphylium</i> sp.
<i>Fusicoccum</i> sp.	<i>Verticillium albo-atrum</i>

The immunity of *Physalospora rhodina* (Berk. and Curt.) Cke. (*Diplodia natalensis* Evans) is of particular interest because of its close morphological similarity to *P. glandicola* and *P. obtusa*. Five isolates of this species were available for testing, two from oak in West Virginia, one from peanut in Alabama, and two from elm in Kansas. This species was tested many times under a variety of conditions but it never supported the growth of the parasite. It will be discussed more completely in the section on resistance.

SPORE GERMINATION

As a result of experiments on nutritional needs for germination, it was evident that an external factor, other than water and oxygen, is required for germination. Spores of *C. parasiticum* failed to germinate or even to swell in water or on water agar. On a glucose-yeast extract agar medium the spores swelled and more than 50 per cent produced germ tubes within 24 hours. The percentage increased during the next 24 hours.

The usual method of germination was by the production of a short germ tube bearing a few secondary spores slightly smaller than the primary spore (Fig. 9). The production of spores continued for several days on a favorable medium, even without a host. A small percentage of the spores gave rise to longer germ tubes with delayed formation of secondary spores. No further development occurred on unsupplemented media until after contact with a susceptible host was made. Minor differences were found in the germination of spores of two different isolates (No. 1012 and 1198). Isolate 1198 was characterized by a somewhat lower percentage of germination and a greater number of long germ tubes with delayed sporulation. This isolate was used in the experiments reported in this bulletin, unless specifically stated otherwise.

As high as 50 per cent germination was reached in the presence of certain natural products in the medium. Some of these products were yeast extract, malt extract, wheat germ, green rose leaf, apple pulp, and mycelial extracts of *Physalospora obtusa*, *P. glandicola* and *P. rhodina*. Germination as high as 25-40 per cent was obtained on agar media containing Casamino Acids, enzymatic hydrolyzed casein and Bactopeptone. Few or no spores germinated on water agar to which each of the following substances was added singly: potassium acetate, glucose, glutamic acid, thiamine, biotin, coprogen, guanine hydrochloride, amino uracil, soybean protein, rolled oats, oak bark and several amino acids. Spores germinated well (80 per cent or above) in the presence of living mycelium of a host and some non-host fungi. Under this condition there was a greater tendency to produce long germ tubes with delayed sporulation.

On the basis of these results it is concluded that one or more essential spore-germination factors are present in many natural materials and are produced by growing mycelium of the hosts and of some non-host fungi. It is evident that a more thorough study of germination factors is needed and a continuation of these investigations is planned.

Germination occurs most rapidly on a suitable medium within a temperature range of 25°-35° C. Within this range germination was 40 per cent or greater within 24 hours. No germination occurred at 40° C.

The principal effect of lower temperatures was to delay germination. When spores of both *C. parasiticum* and *P. obtusa* were placed together on plates of malt extract-yeast extract agar, germination and parasitism were evident within seven days at temperatures as low as 10° C. About 10 per cent of the spores that had been frozen continuously in distilled water for 12 months germinated on a favorable medium at 25° C.

TROPISM OF HOST HYPHAE TOWARD GERMINATED SPORES OF PARASITE

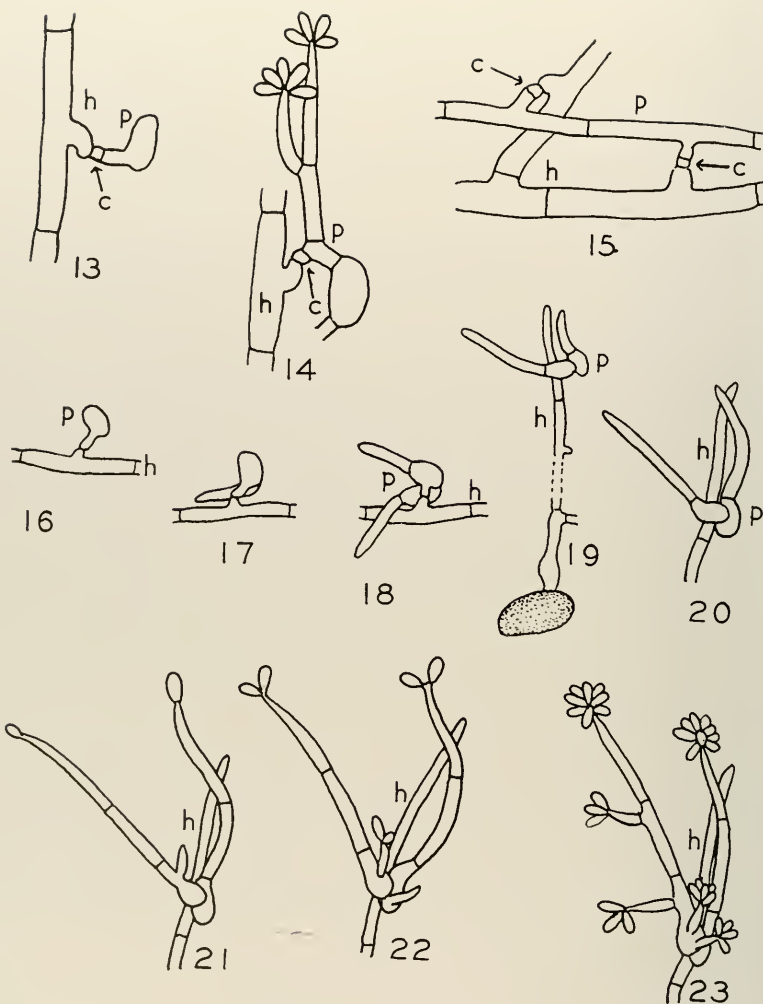
During the study of spore germination of *C. parasiticum* in the presence of the host it was observed many times that contacts between parasite and host are not always due to chance. Host hyphae growing among scattered spores of the parasite were stimulated to send out short lateral branches toward germinating spores, or less often the tip of the main hypha would curve from its normal course and grow directly toward the parasite. The stimulus is assumed to be a chemical substance secreted by the germinating spores. The strength of the reaction, based on distance between host and parasite, appears to vary with the stage of germination, the medium and the species of host. In one case, observed at intervals through the microscope, the host hypha 40 microns away began to turn toward germinated spores of the parasite and required 4 hours to cover the intervening distance and make contact (Figs. 10, 12). There is also evidence that when the host comes within a few microns of a parasite spore the latter may put out a short tube that grows toward the host.

A dilute glucose-yeast extract (3-1) agar medium was satisfactory for observing tropism of host hyphae. At this dilution and on water agar the mycelium was sparse enough so that it could be studied easily with the microscope. The hyphae of *P. obtusa* reacted somewhat more strongly than did *P. glandicola*. It is of particular interest that *P. rhodina*, an immune species, showed as great a tropism toward the parasite as did the highly susceptible hosts.

The chemical stimulator is active in high dilution and it is highly potent. The host hyphae attracted to the parasite are frequently wider than normal and produce several short knob-like branches. Sometimes the germ tubes from 2 or 3 parasite spores may anastomose, being directly attracted to each other.

MODE OF PARASITISM

The actual contact between the parasite and host is usually accomplished by means of short lateral branches only a few microns long which touch at their tips (Figs. 13-15). The mode of parasitism of *C. parasiticum* appears to be unique. The wall at the point of contact is flattened, but



Figures 13-23, *Calcarisporium parasiticum*. The buffer cell produced at the point of host-parasite contact and stages in the development of the parasite from the time of host contact until sporulation. (13) The buffer cell (c) at the point of contact between germ tube of the parasite (p) and short lateral branch of the host (h). (14) Similar to 13 but after further development. (15) Buffer cells (c) between host (h) and parasite (p) at the frequent contacts of the mycelia. (16-18) Time-lapse drawings of the same parasite (p) from the time of contact till the production of two hyphae, covering a period of six hours. (19-23) Time-lapse drawings of a parasite (p) from the stage shown in 18 till the production of several clusters of conidia, covering a period of 22 hours. The parasite (p) is attacking a germ tube of *P. obtusa* (h) growing on water agar. Note that no growth of the host hypha (h) occurs during this period.

there is no penetration of the host either by haustoria or by non-specialized branches. Yet, a satisfactory nutritional relationship is established immediately following contact and the parasite begins to develop rapidly within a few hours. This suggests that certain essential nutrients present in the host mycelium pass freely from host to parasite. This movement may be initiated or stimulated as a result of increased permeability of the host membrane as a response to the parasite. There is no evidence of any harmful effect on the host, except a reduction in the rate of growth. *C. parasiticum* is, therefore, a good example of the balanced type of parasitism.

The actual area of contact between parasite and host is small, usually no more than 2 microns in diameter. In most cases there appears to be a small cell about 2 microns wide formed at the tip of the parasite hypha where it contacts the host (Figs. 13, 15). This tiny "buffer" cell is not always visible at contact points, possibly due to the position of the hyphae, but it is believed to occur in all or nearly all cases.

The function of this contact cell is purely a matter of speculation at present, but it appears to facilitate a successful parasitic relationship. It possibly functions to increase the permeability of the host cell membrane and to absorb the essential nutrients from the host. It appears to differ from the buffer cell produced between *Chaetocladium* and *Parasitella* and their hosts (Burgess, 1924). There is no evidence of any dissolution of the cell wall by *C. parasiticum*. The spread of the parasite over the host colony is accompanied by frequent contacts with the mature cells of the host (Fig. 15). These contact points can be recognized by the presence of the small buffer cell described above. No buffer cells have been observed at contact points between *C. parasiticum* and *P. rhodina*.

If one assumes that contact between host and parasite hyphae occurs as a result of a chemical stimulus secreted by one or both fungi, it is reasonable to conclude that this substance might be flushed away or at least greatly reduced by a constant flow of liquid medium over the cultures. To test this idea, spores of the parasite in a water suspension were distributed over the surface of plates of malt extract yeast extract agar and a bit of mycelium of *P. obtusa* placed at one edge. After a period of 24 hours, which permitted the hyphae to become anchored in the agar, a sterile liquid medium containing 1 g. of malt extract and 0.5 g. of yeast extract per liter was dripped slowly and continuously onto the culture and allowed to flow over the edge of the specially adapted Petri dish. After 2 days the host mycelium was examined microscopically for contacts with the parasite and for anastomoses between

host hyphae. These were compared with the frequency of contacts on agar medium with no treatment. The continuous flow of liquid medium greatly reduced but did not completely eliminate the number of parasitic contacts. Likewise it reduced the number of anastomoses between host hyphae.

GROWTH AND DEVELOPMENT OF THE PARASITE

One of the striking characteristics of *C. parasiticum* is the rapidity of its development on a highly susceptible host. For the study of the early development of the parasite either water agar or a highly diluted malt-yeast medium was selected because a sparse growth of the mycelium is essential to clear microscopic examination of early development. A mixture of spores of the parasite and host was used as inoculum. A number of areas on the plates were marked and examined at intervals to follow the rate of development. Stages in development were recorded in a series of drawings (Figs. 16-23). Figures 16-18 show the same individual from the time of contact to the production of two separate branches 6 hours later. Figures 19-23 show a sequence of development of another individual on a hypha from a germinating spore of *P. obtusa*. Under these conditions the parasite produced its first spores about 12 hours after contact with the host and within 24 hours it had produced several clusters of conidia.

Figures 19-23 illustrate clearly one of the principal effects of *C. parasiticum* on its host. The point of contact in this case (hidden by the parasite in the drawings) was near the tip of the host hypha growing on water agar. During the 23-hour period covered in this sequence the parasitized hypha made no further growth. Yet, it provided the young parasite with sufficient nutrients within this period to develop rapidly and produce six sporogenous cells, each bearing a cluster of conidia. It was apparent that the parasite absorbed through its one point of contact with the host sufficient nutrients for rapid development and in doing so deprived the host of nutrients needed for its own growth. This suggests the possibility that the key material needed by the parasite is also essential to the host.

Stages in the development of the conidiophore of *C. parasiticum* are shown in Figures 5, 21-23. The conidiophore first appears as a slender, simple hypha, the apical cell of which may soon produce spores. In the meantime the conidiophore sometimes becomes branched. Other sporogenous cells arise as side branches from the main axis of the conidiophore. If the conidiophore hypha is short, only one to a few sporogenous cells will be produced, but if it is made up of several cells, a large number of sporogenous cells may be produced. It is not clear what factors determine the size of the conidiophore and number of sporogenous cells,

but it seems certain that nutrition is an important factor. Early formation of conidiophores is frequent but does not always occur. After 2 or 3 days the mycelium of the parasite can be seen growing slowly outward among the hyphae of the host making contact where they cross.

The effects of temperature on growth and development of *C. parasiticum* were determined using *P. obtusa* as the host. Plates of malt extract-yeast extract agar were inoculated at three points with mycelium of the host and a drop of parasite spore suspension added. The cultures were incubated at 5° intervals in temperature from 5° to 40° C. Both the host and the parasite developed within the range of 10° to 35° C., with the optimum about 25° to 30° C., near the optimum of the host. There was poorer development of the conidiophores at 35° than at 30° C., but the number of spores was nearly as great. The principal effect of temperatures below optimum was to reduce the rate of growth of both host and parasite, but a few spores were produced at 10° C.

Experiments to determine the effects of the carbon source in the host medium on the growth of the parasite were conducted on sugar-glutamic acid (3-1) agar media. Both *P. obtusa* and *P. glandicola* were used as hosts. The development of the parasite was essentially the same on both hosts and was not proportional to the growth of the host. Development and sporulation of *C. parasiticum* were best on glucose, fructose, galactose, maltose and sorbose, and poorer on lactose and on medium without sugar. Host growth was very slow on sorbose, but with *P. obtusa* in particular, the parasite completely covered the host mycelium.

The effects of the nitrogen source in the host medium on the development of the parasite were tested using the glucose-nitrogen source (10-2) agar media. The initial pH of each medium was 6.0. The rate of development of the parasite varied with the medium and the host. Observations made on the tenth day were recorded as trace, poor or sparse, good but with little spread, and excellent and spreading (represented by classes 1, 2, 3 and 4, respectively. Results are shown in Table 2.

EFFECT OF PARASITE ON GROWTH OF HOST

It was evident from both microscopic observation and macroscopic appearances that *C. parasiticum* reduces the rate of growth of the susceptible host within the area occupied by the parasite. However, it is difficult to measure actual rate of growth and the effect of the parasite on agar media. Preliminary experiments showed that the parasite not only affected the extension of the mycelium but also reduced the dry weight of mycelium produced by the host. Since it was not possible to separate host and parasite mycelium it was necessary to use the total growth of both, with the realization that the actual reduction in weight

TABLE 2. GROWTH OF 2 ISOLATES OF *C. parasiticum* ON 3 HOSTS GROWN ON DIFFERENT NITROGEN SOURCES. DATA ARE FROM 10-DAY-OLD CULTURES

NITROGEN SOURCE	PARASITE ISOLATE 1012 ON			PARASITE ISOLATE 1198 ON		
	P. GLANDICOLA		P. OBTUSA	P. GLANDICOLA		P. OBTUSA
	No. 1138	No. 1199	No. 1084	No. 1138	No. 1199	No. 1084
KNO ₃	3*	3	3	4	4	4
Ammonium sulfate	1	1	2	2	2	2
Ammonium sulfate and CaCO ₃	1	2	2	2	2	2
Ammonium tartrate	2	3	3	3	4	4
Asparagine	3	3	3	3	4	4
Glutamic acid	3	3	2	3	4	4
Phenylalanine	2	2	3	3	3	4
Casamino Acids	2	3	3	3	4	4

* 1 = trace; 2 = poor, sparse; 3 = good but little spread; 4 = excellent and spreading over host.

of the host was greater than shown by the differences between the dry weights of the host alone and the host-parasite combination.

An additional effect of the parasite on the highly susceptible host was evident when an agar medium was inoculated with a bit of host mycelium and a drop or two of spore suspension of the parasite. The latter spread out and covered an area about 10 mm. in diameter. As the host grew outward through this area the mycelium was quickly parasitized and failed to develop the dark melanin pigment characteristic of *Physalospora* (Fig. 2). The portion of the host mycelium that extended beyond the parasite soon became dark. The parasite later overgrew the host but did not destroy the pigment in the dark mycelium.

The nitrogen sources in agar media on which the hosts were grown affected the development of the parasite (Table 2). In general the development of the parasite was poor when ammonium sulfate, with or without calcium carbonate, was used and good to excellent on other nitrogen sources. The parasite isolate No. 1198 was somewhat more virulent than isolate No. 1012.

It was also desirable to determine if growth in liquid would be similarly affected. *P. glandicola* (Isolate No. 1138) was chosen as the host and isolate 1198 of the parasite was used. The media contained 10 g. glucose and 2 g. of nitrogen source per liter. The initial pH of all media was 6.0. The dry weights with pH reading at all harvests are shown in Table 3.

In all media, except ammonium sulfate without CaCO₃, the total dry weight of host and parasite was less than that of the host alone under the same conditions. It is possible that the nitrogen sources may affect the growth of the parasite directly, but on the basis of observation the direct effect on growth of the parasite alone within the 10-day period of this experiment would be a minor contributing factor. (See a later

TABLE 3. WEIGHT OF MYCELIUM AND pH OF CULTURE FILTRATES OF *P. glandicola* (ISOLATES 1138) ALONE AND WITH *C. parasiticum* (1198) IN LIQUID MEDIA CONTAINING DIFFERENT NITROGEN SOURCES

HOST ALONE	KNO ₃		AMMONIUM SULFATE		AMMONIUM SULFATE & CaCO ₃		AMMONIUM TARTRATE		PHENYLALANINE		CASAMINO ACIDS	
	mg.	pH	mg.	pH	mg.	pH	mg.	pH	mg.	pH	mg.	pH
4 days	47	6.2	29	2.3	105	3.5	61	3.6	17	3.5	114	4.3
6 days	91	7.1	28	2.6	132	3.0	114	3.3	48	3.2	127	4.2
10 days	100	7.7	36	2.4	112	3.2	98	3.6	67	3.6	109	5.1
Host and Parasite												
4 days	18	5.7	52	3.0	54	5.4	27	4.6	9	4.1	60	4.3
6 days	33	5.9	43	2.8	79	2.8	59	3.7	25	3.7	100	3.7
10 days	60	6.9	40	2.7	97	5.5	87	4.1	53	3.7	100	5.7

section for method of growing *C. parasiticum* in absence of living host.)

The effect of the parasite on the host in liquid media is to reduce the rate of growth rather than to check growth entirely after a few days (Table 3). Inhibition is somewhat greater in a potassium nitrate medium, which allows slower growth of the host than does the hydrolyzed casein medium. Therefore, rapid growth of the host is not essential to vigorous parasitism. The greater weight of the host-parasite mixture in the ammonium sulfate medium without calcium carbonate may have been due to the slightly higher pH for the host-parasite culture medium. It is believed that the minimum pH for the host is around 2.4. However, within a more favorable range such a slight difference in pH had no visible effect. Both the host and the parasite developed well within the pH range of 3.2 to 7.0. In general the presence of the parasite had little effect on the pH changes in the media.

At this time it became desirable to determine whether the isolates of *C. parasiticum* were similar in virulence and effect on the host. Four isolates (No. 1012, 1198, 1214 and 1222) were then compared on *P. obtusa* (isolate No. 1081) on two media, one with 2 g. of potassium nitrate and the other with 2 g. of Casamino Acids per liter as the nitrogen sources. The results on the Casamino Acids medium are presented graphically in Figure 24, and on the potassium nitrate medium in Figure 25. The most outstanding results show a family of curves representing growth of host-parasite cultures well below the curve for the host alone. Growth was more rapid on the Casamino Acids medium but the curves are no farther apart. The greatest differences were evident at the eight-day harvest. At this time the parasite isolate 1012 showed the least virulence (judged by the least depression of the growth of the host) and 1198 was the most virulent, but the difference throughout the 17-day period of the experiment was not great.

Although much information was gained in the above experiments,

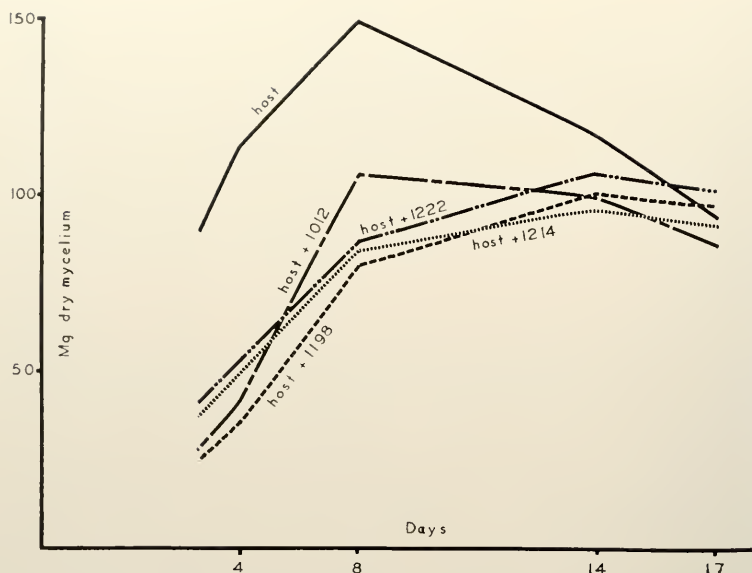


Figure 24. Growth of *P. obtusa* (isolate 1084) alone and in combination with each of four isolates of *C. parasiticum* in a glucose-Casamino Acids medium.

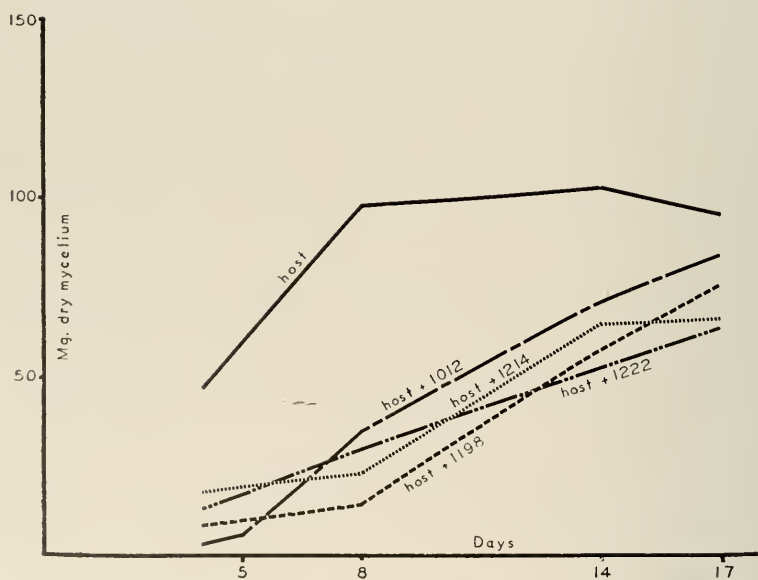


Figure 25. Growth of *P. obtusa* (isolate 1084) alone and in combination with each of four isolates of *C. parasiticum* in glucose-potassium nitrate medium.

it was still necessary to learn more about the important factor of time and to follow the cultures more closely during the growth period. More extensive experiments were conducted, following the growth of *P. glandicola* (No. 1138) on Casamino Acids and potassium nitrate media, with and without the parasite (No. 1198). The results are shown by growth curves in Figure 26, and pH changes of the medium during growth are shown in Figure 27.

The inhibitory effects of the parasite are striking by the fourth day on both media. At any given time through the 20th day the weight of the host-parasite culture was less than that of the host. This was true on both media. From observation, growth of the parasite was approximately equal on both media, but the proportion of parasite to host was greater on the nitrate medium. It was not possible to determine whether the parasite reached a maximum weight, but it appeared that the parasite continued to grow and sporulate well beyond the point at which the host reached maximum weight.

Duplicate experiments were carried out simultaneously using *P. obtusa* (No. 1084) as the host. The growth curves are presented in Figure 28 and the pH changes in Figure 29. In general the results are similar to those when *P. glandicola* was the host, but even greater effects of the parasite are evident. The decline in weight of *P. obtusa* with the parasite on Casamino Acids medium is greater than that of *P. glandicola*. The increased spread of the curves of cultures on Casamino Acids is believed to be due to increased autolysis in the host-parasite cultures and to continued activity of the parasite.

As a comparison three other isolates of *P. obtusa* from different hosts were inoculated with *C. parasiticum* on a potassium nitrate medium. Growth curves of cultures with and without the parasite are shown in Figure 30. Isolate 1130 was obtained from oak, 1166 from red bud (*Cercis*) and 1142 from honey locust, all from West Virginia. Growth curves of isolates 1130 and 1166 are very similar and both are reduced sharply by the presence of the parasite. The growth curve of 1130 is more nearly like that of isolate 1084 from apple (compare with Figure 28). Isolate 1142 differed from the others. It grew rapidly for 7 days and then slowly began to autolyze. The presence of the parasite had much less effect on 1142 than on any of the other three isolates. Thus, it may be concluded that isolate 1142 is more resistant to *C. parasiticum* under these conditions.

The effects of the growth of the parasite on three additional isolates of *P. glandicola*, all from oak in West Virginia, were determined under the same conditions. The growth curves are shown in Figure 31.

In a similar way the effects of growth of the parasite on two isolates of *Botryosphaeria ribis* were determined. Growth curves are shown in

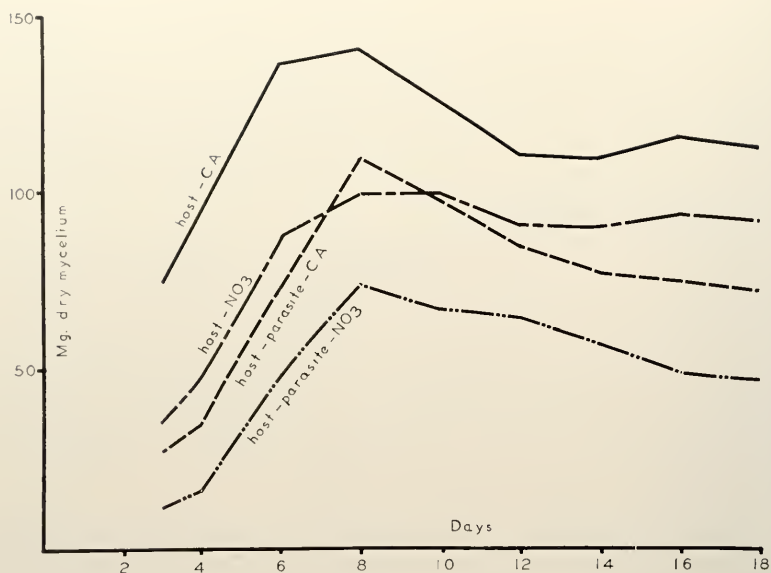


Figure 26. Growth of *P. glandicola* (isolate H138) alone and with *C. parasiticum* in glucose-Casamino Acids and glucose-potassium nitrate media.

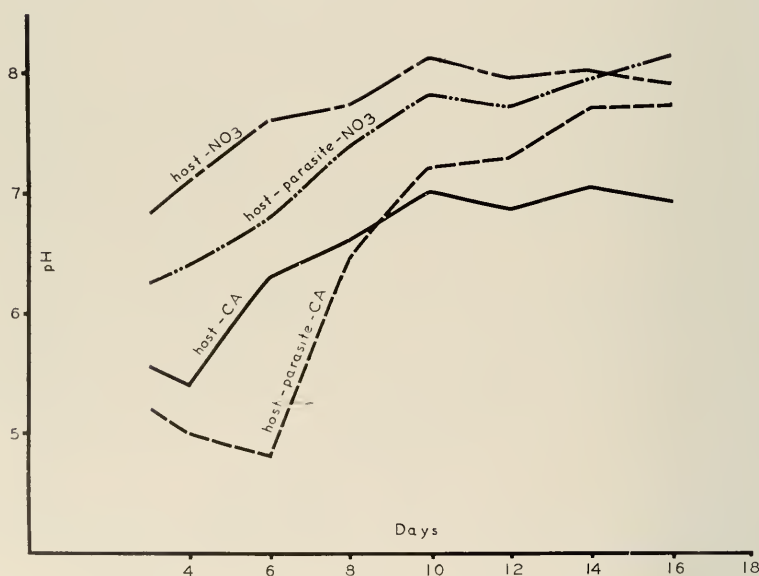


Figure 27. The pH of the filtrates of the same cultures reported in Figure 26.

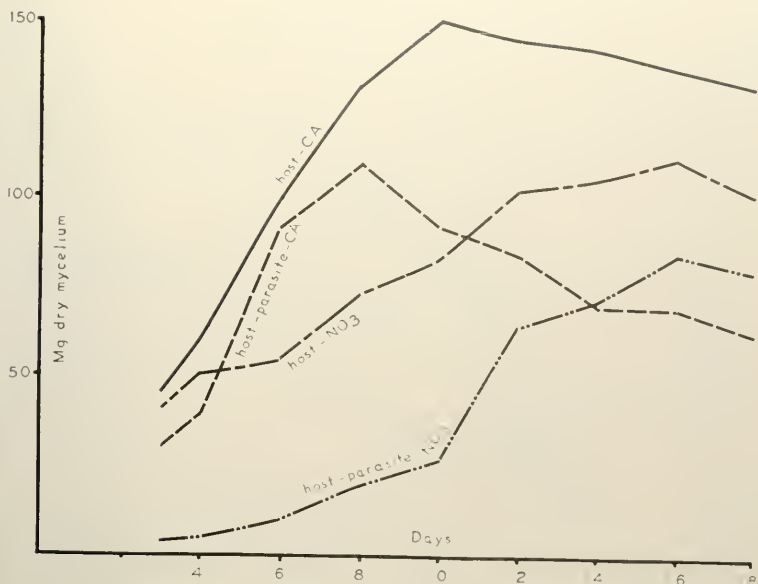


Figure 28. Growth of *P. obtusa* (isolate 1081) alone and with *C. parasiticum* in glucose-Casamino Acids and glucose-potassium nitrate media.



Figure 29. The pH of the filtrates of the same cultures reported in Figure 28.

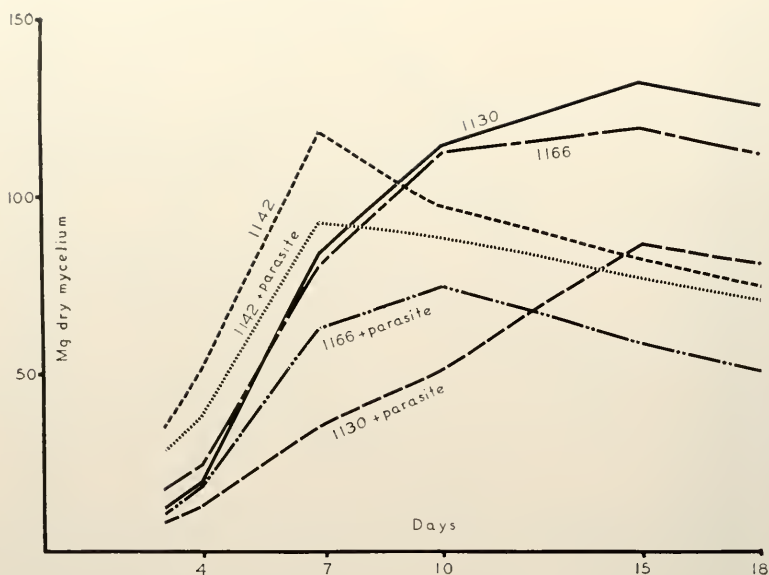


Figure 30. Comparison of growth of three additional isolates of *P. obtusa* alone and with *C. parasiticum* in a glucose-potassium nitrate medium.

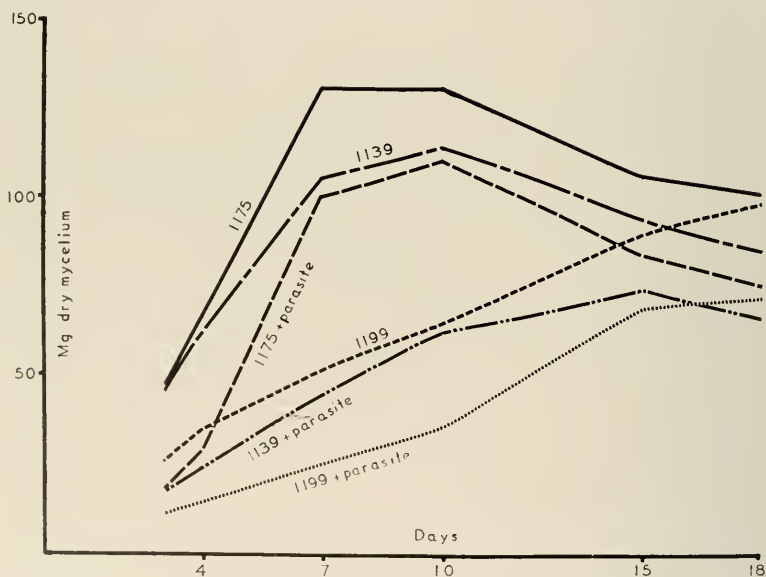


Figure 31. Comparison of growth of three additional isolates of *P. glandicola* alone and in combination with *C. parasiticum* in a glucose-potassium nitrate medium.

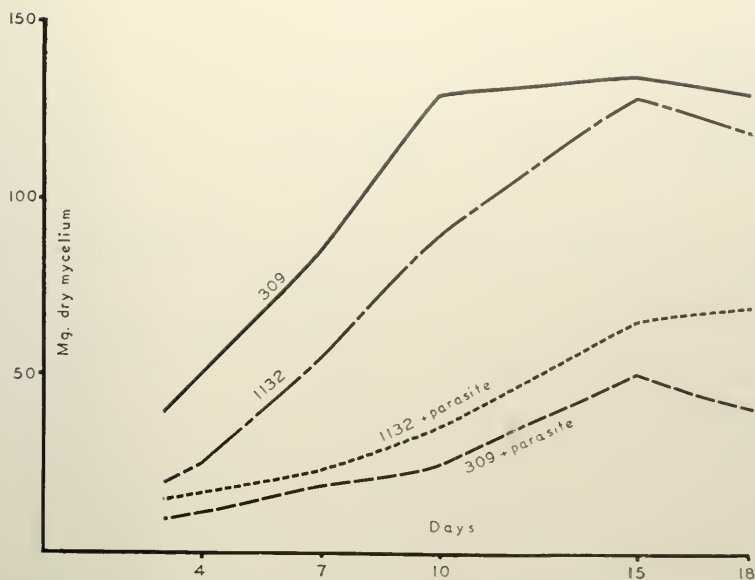


Figure 32. Comparison of growth of two isolates of *Botryosphaeria ribis* alone and in combination with *C. parasiticum* in a glucose-potassium nitrate medium.

Figure 32. Isolate No. 1132 was obtained from oak and No. 309 from apple. The response of these two isolates was similar and much like that of other species of host.

A few general conclusions may be drawn as a result of studying in some detail the responses of a total of 10 isolates from three highly susceptible species of host. Isolates from the same species (*P. glandicola*) of host tree may vary considerably in their response (resistance) to *C. parasiticum*. There was sometimes more difference between isolates of the same species than between host species (compare *P. obtusa* and *B. ribis*). Growth of all host isolates tested was depressed by the presence of the parasite under these conditions. The degree of severity of parasitism was affected by the host medium as well as the host species. There was little difference in the virulence of isolates of *C. parasiticum*.

It has been noted above that the growth rate of the host, particularly *P. obtusa*, is less on a medium containing nitrate nitrogen than on one containing Casamino Acids, yet the host mycelium growing on both media was parasitized equally. It had been concluded tentatively that rapid growth of the parasite is not essential to heavy parasitism. However, it was desirable to put this theory to a more severe test. It was also known from previous work (Lilly and Barnett, 1953) that *P. obtusa* grows slowly on a medium containing sorbose as the only carbon source. Two liquid media were then used, each containing 10 g. sorbose per liter.

and 2 g. of Casamino Acids or 2 g. of potassium nitrate per liter. Inoculation was by mycelium of host and spores of the parasite. The parasite sporulated heavily on the host mycelium on both media as soon as it reached the surface. Small submerged colonies of the host also were heavily parasitized and the parasite was sporulating. Dry weights of these cultures are presented in Table 4.

TABLE 4. COMPARISON OF GROWTH OF *P. obtusa* ALONE AND WITH THE PARASITE ON TWO MEDIA CONTAINING SORBOSE. DRY WEIGHTS ARE GIVEN IN MG. PER CULTURE

MEDIUM	DAYS	HOST ALONE	HOST AND PARASITE
Sorbose-nitrate	21	53	10
Sorbose-nitrate	36	86	14
Sorbose-Casamino Acids	21	111	42
Sorbose-Casamino Acids	36	91	72

Even at the end of 36 days the host mycelium alone had not reached maximum weight on the sorbose-nitrate medium, but maximum weight had been reached on the sorbose-Casamino Acids medium. The parasite caused severe inhibition of host growth on both media, being greater on the nitrate medium, where little total weight was produced. Microscopic examination showed that nearly all of the mycelium in the nitrate medium belonged to the parasite and only a little host mycelium was present. In the Casamino Acids medium the host was heavily parasitized but it still made greater growth than on the nitrate medium. This indicates that only a small amount of host mycelium is necessary for continued slow growth of the parasite.

SUSCEPTIBILITY AND RESISTANCE TO THE PARASITE

In the host range studies reported above it was found that all tested isolates of *P. obtusa*, *P. glandicola* and *B. ribis* were highly susceptible to *C. parasiticum*. Only minor variation in degree of susceptibility was noted between isolates of the same species under uniform conditions and there was little difference between these three species of hosts. When 3-week-old cultures of these highly susceptible species were inoculated by placing drops of a spore suspension of the parasite on the older portions of the mycelium, the parasite began to grow within a few days and slowly spread over the host mycelium. In another test cultures of *P. obtusa* were grown for 7 days (near maximum weight) in glucose-Casamino Acids liquid medium. The mycelium was washed thoroughly and placed in sterile distilled water for as long as 30 days. In all tests the aged, starved mycelium proved to be susceptible and was readily parasitized by *C. parasiticum*.

These experiments show that a compatible nutritional relationship can be established by the parasite even with old mycelium and that the nutrients required by the parasite are present in the old and young mycelium alike. Neither growing cells nor a high degree of metabolic activity is essential to parasitism. Maturity of cells of the mycelium of these susceptible species is not a factor in resistance.

In considering possible ways by which the host fungi could be made more resistant or more susceptible, the nitrogen source and concentration were among the most likely to be of importance. It was shown above that there was no great difference in the effects of several nitrogen sources in approximately the same concentrations. Experiments were then designed to determine whether the concentration of amino acids or the carbon-nitrogen ratio in the host medium would alter the degree of parasitism by *C. parasiticum*. Liquid media were made up containing glucose and Casamino Acids in the following amounts in g. per liter: 20-1, 10-2, 10-10, and 5-10. *P. obtusa* was used as the host fungus. The results of one experiment are shown in Figure 33.

The greatest amount of growth of the host alone (210 mg) was in the 20-1 medium, which allowed only little growth of the parasite as judged visually. This is also reflected in the relatively slight depression in the total weight of host and parasite grown together. A reduction in

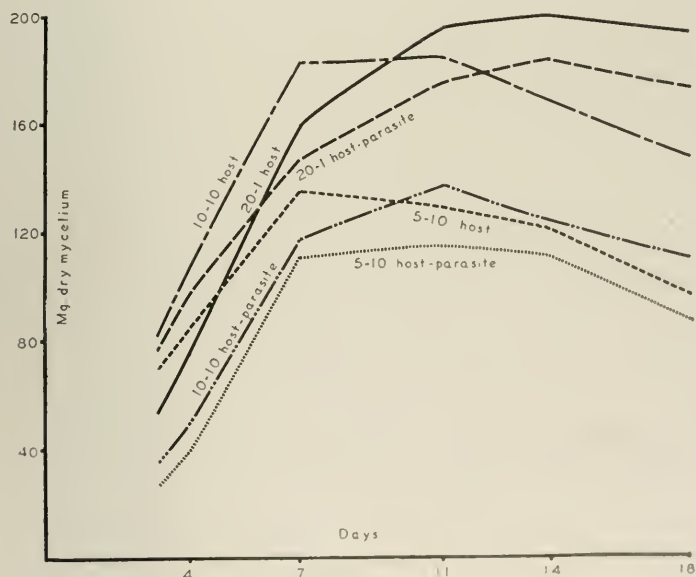


Figure 33. Comparison of growth of *P. obtusa* alone and in combination with *C. parasiticum* in three media varying in amounts (g. per liter) of glucose and Casamino Acids.

glucose to 10 g. and an increase in Casamino Acids to 2 g. (not shown on graph) resulted in a decrease in maximum weight of host alone to 135 mg. The parasite made greater growth and caused a greater reduction in weight of the host-parasite cultures than on the 20-1 medium. The 10-10 medium allowed greater maximum growth of the host alone (193 mg.) than did the 10-2 medium, presumably because of the added carbon in the Casamino Acids. This medium permitted excellent growth of the parasite, a fact also reflected in the great decrease in weight of the host-parasite cultures as compared to the host alone. The two media containing 10 g. of Casamino Acids per liter were surprisingly similar in the growth of the host and parasite together, regardless of the 2-fold difference in the amount of glucose present. Growth of the parasite on *P. obtusa* was excellent in both media and most of the final weight in the 5-10 medium is believed to be due to the parasite.

The effects of concentration of amino acids on resistance of *P. ilicis* and *Coniothyrium* sp. to *C. parasiticum* were tested on agar media using lower concentrations of glucose and Casamino Acids. Agar media were used because these 2 host species were more resistant than *P. obtusa* and it was likely that they would show little or no depression of weight in liquid culture. These media contained the following proportions of glucose and Casamino Acids: 20-1, 10-1, 3-4, and 3-8. The agar plates were inoculated at three points with host mycelium and a spore suspension of the parasite was added to two of these areas. The amount of growth of *C. parasiticum* was estimated visually and classed in 4 groups ranging from 1 (slight; host highly resistant) to 4 (excellent; host highly susceptible). No visible growth of the parasite was recorded as negative. The results are summarized in Table 5. The results confirmed previous observations on other media that *P. obtusa* is highly

TABLE 5. ESTIMATED RELATIVE GROWTH OF *C. parasiticum* ON THREE HOSTS CULTURED ON FOUR AGAR MEDIA DIFFERING IN CONCENTRATIONS OF GLUCOSE AND CASAMINO ACIDS. RESULTS ARE RECORDED IN FIVE GROUPS: 0 (NONE), 1 (SLIGHT, HOST HIGHLY RESISTANT) TO 4 (EXCELLENT, HOST HIGHLY SUSCEPTIBLE)

HOST AND TIME	20 GL.-1CA	10 GL.-1CA	3 GL.-1CA	3 GL.-8CA
<i>P. obtusa</i>				
5 days	2	2	2	3
10 days	4	4	1	4
<i>P. ilicis</i>				
5 days	0	0	1	1
10 days	1	2	4	4
14 days	1	2	4	4
<i>Coniothyrium</i> sp.				
5 days	0	0	0	0
10 days	0	0	1	1
14 days	0	0	1	2

susceptible. *P. ilicis* is intermediate in resistance, and *Coniothyrium* sp. is highly resistant. While *P. obtusa* differed little in degree of susceptibility on these media, both *P. ilicis* and *Coniothyrium* sp. were decidedly more resistant on media containing a high carbon-nitrogen ratio. Both species showed the greatest susceptibility on media high in nitrogen and low in sugar. A comparison of resistance of two hosts in different media is shown in Figures 34-41.

Thus, the results of these experiments confirm the theory that the degree of resistance of the hosts of *C. parasiticum* can be modified by changing the concentration of amino acids and the sugar in the medium. It is assumed that the mycelium grown on a higher concentration of amino acids contains within its cells a higher concentration of soluble nitrogenous compounds. It is possible that this is the basis for the degree of susceptibility of the hosts. The validity of this theory must await further study of the nitrogen composition of the host mycelium, both quantitative and qualitative.

GROWTH OF PARASITE IN THE ABSENCE OF A LIVING HOST

It was reported above that little or no germination of spores of *C. parasiticum* occurred on water agar. Later it was discovered that the addition of a drop of water extract of the mycelium of *P. obtusa* to spores on water agar resulted in 75 per cent germination within 24 hours. Addition of the extract to spores on glucose-yeast extract agar also increased germination and resulted in the production of longer germ tubes that continued to grow slowly after some sporulation had occurred. More mycelium was produced than under any other condition in the absence of the living host. Repeated transfers from spores or mycelium and spores to fresh medium with added host extract resulted in no decrease in germination or in the growth of cultures. In the absence of the host extract, development of the parasite did not go beyond the production of spores and a few hyphae on a yeast extract medium as described above under spore germination.

Until the extract of host mycelium was added to transfers of *C. parasiticum* this species was thought to be an obligate parasite. However, it was now clear that the parasite was able to absorb from its medium all of the nutrients required for continued slow growth and sporulation through repeated transfers away from the living host. But none of the media tried, even with the addition of the mycelium extract, has permitted the normal, rapid development that takes place on mycelium of a living host. Pure cultures of *C. parasiticum* on glucose-yeast extract agar with added host extract continued to sporulate on the surface and within the agar, producing colonies in tubes that appeared somewhat yeast-like. Only imperfectly-formed conidiophores were present. None was truly verticillate.

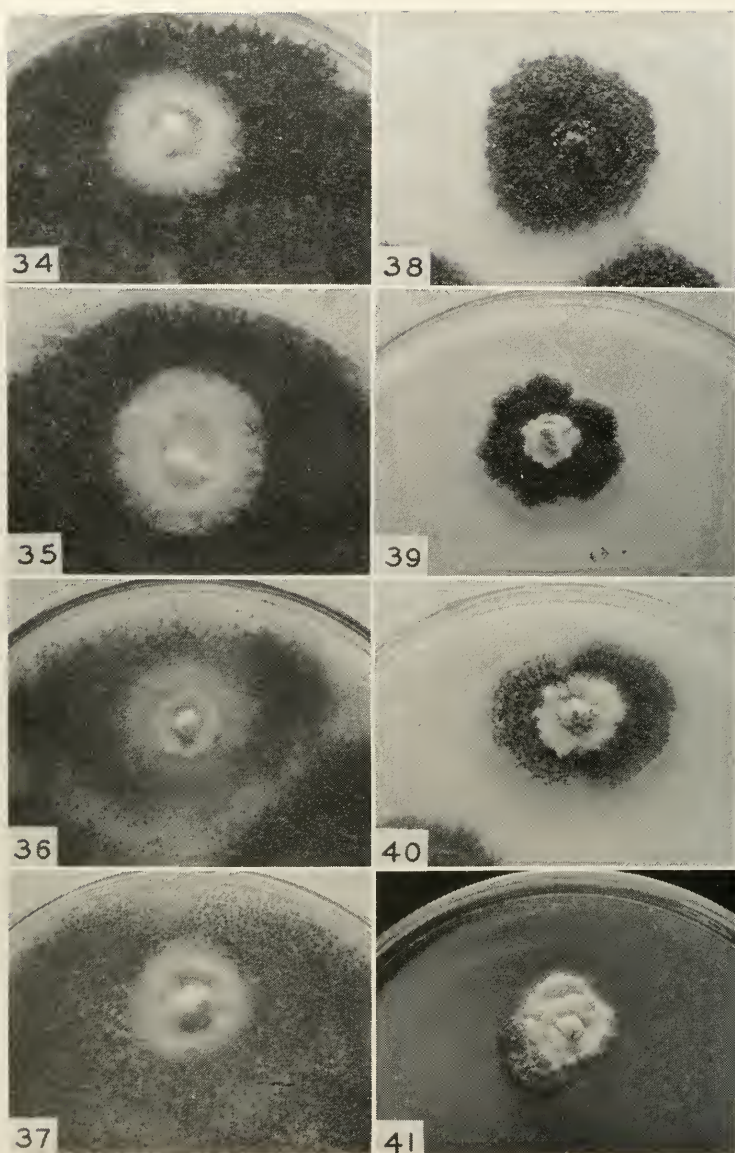


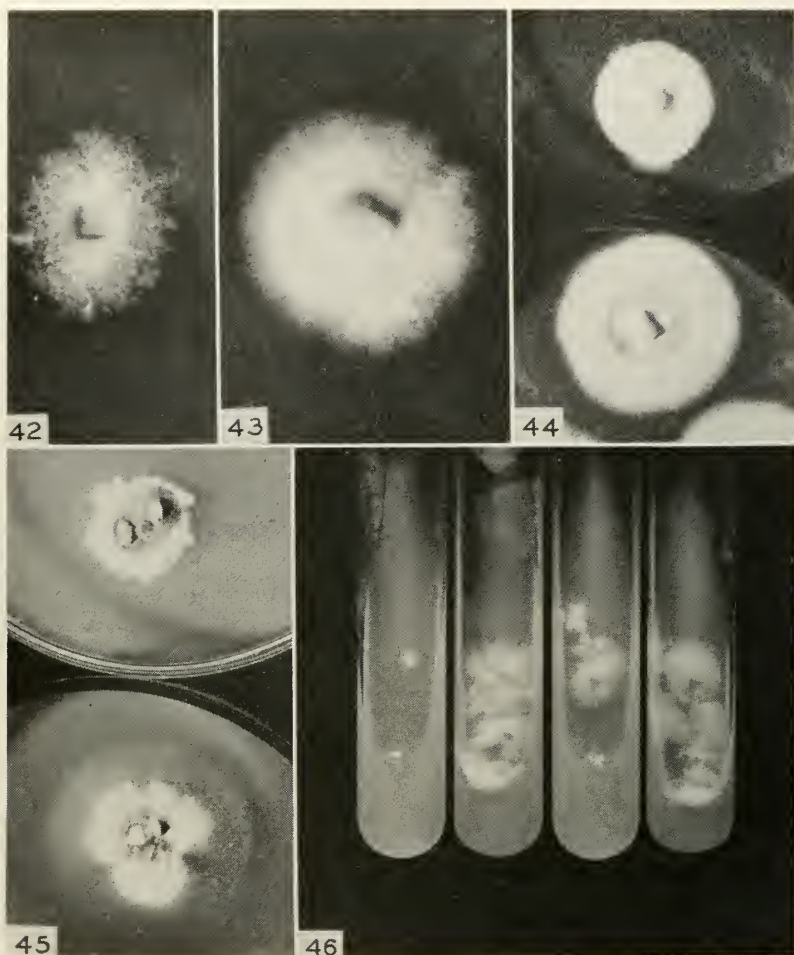
Figure 34-41. The effects of concentrations of glucose and Casamino Acids in the host medium on the growth of *C. parasiticum* on *P. obtusa* (34-37), a highly susceptible host, and *P. ilicis* (38-41), a resistant host. Reading from top to bottom the amounts per liter of glucose and Casamino Acids are 20-1, 10-2, 5-4, and 5-8. Note that there is little difference in susceptibility of *P. obtusa*, but *P. ilicis* is much more resistant on the high carbon-low nitrogen medium. Cultures were 12 days old.

There appeared in the pure cultures several small colonies of *C. parasiticum* which produced more mycelium and appeared less yeast-like than the parent culture (No. 1198). Transfers made from one of these small mycelial colonies developed into the same type of growth and subsequent cultures remained uniform. It was concluded that this variant represented a mycelial type mutant which was designated at 1198-M. The mutant grew somewhat more rapidly than did the parent culture and it produced more mycelium and fewer spores (Figs. 42, 43).

The mutant was then tested for pathogenicity on cultures of *P. obtusa* and compared with the parent culture and with the parasite taken directly from the host. There was little difference in the rate of growth of the parasite originating from the three sources of inoculum. After 7 days the average diameters of the parasite colonies were 23, 22 and 19 mm., respectively, following use of inoculum directly from the host, from a 5-month-old pure culture of 1198, and from the pure culture of the 1198 mutant (Fig. 44). Thus, neither the continued growth of *C. parasiticum* in pure culture nor the mutation had any appreciable effect on pathogenicity.

The use of the mycelial mutant now made it possible to measure more accurately the diameter increase of the parasite colony on agar. Growth of the parasite alone (isolate 1198M), but with added host extract, was greater on a glucose-yeast extract (3-4) medium than on a glucose-yeast extract (20-1) medium (Fig. 45). After 30 days the colonies averaged 35 mm. in diameter on the former medium and 18 mm. on the latter. Growth on the same media with no added mycelial extract was negligible.

The favorable effects of living mycelium and extracts of host and non-host fungi on germination of *C. parasiticum* were reported above. It was of equal importance to know whether the extracts from mycelium of non-hosts or resistant species contained the same growth stimulating factors that were present in extracts of highly susceptible hosts. The fungi chosen were *P. obtusa* (highly susceptible), *P. ilicis* (highly resistant on the medium used) and *P. rhodina* (immune). Extracts were prepared in the usual way from 7- to 10-days-old cultures growing in a glucose-yeast extract (10-2) liquid medium and were sterilized by autoclaving. Plates of glucose-yeast extract (3-4) agar were inoculated at 3 points with *C. parasiticum* and one drop of extract added to two of the inoculation pieces, leaving the third as a check. The parasite made approximately equal growth in the presence of each of the 3 extracts. The colonies averaged 11, 11 and 12 mm. at 7 days and 16, 13 and 17 mm. at 18 days, respectively, for extracts of *P. obtusa*, *P. ilicis* and *P. rhodina*. The effects of the extracts on growth of tube cultures on glucose-yeast extract agar are shown in Figure 46.



Figures 42-46. Comparison of *C. parasiticum* parent and mutant cultures, and growth of the parasite in the absence of a host. (42) Colony of parent culture of *C. parasiticum* on a glucose-yeast medium fortified with one drop of concentrated extract of mycelium of *P. obtusa*, 16 days old. (43) Colony of mutant culture of *C. parasiticum* under the same conditions as the parent culture shown in 42. (44) Comparative growth of the parent culture (bottom) and the mutant (top) of *C. parasiticum* growing on a culture of *P. obtusa*, 12 days old. (45) Comparative growth of mutant culture of *C. parasiticum* on glucose-yeast extract agar at concentrations of 3-4, (bottom) and 20-1 (top), both supplemented with one drop of concentrated mycelial extract of *P. obtusa*. (46) Effect of 2 drops of extracts of three species of *Physalospora* on growth of the mutant of *C. parasiticum* on a glucose-yeast extract medium in the absence of a host. A. No extract; B. Extract of *P. obtusa*; C. Extract of *P. ilicis*; D. Extract of *P. rhodina*. Note that the extract of the immune species is nearly as effective in promoting growth as that of the highly susceptible species. Cultures were 16 days old.

Under no condition tested could the development of *C. parasiticum* in the absence of a living host be considered as normal for the species, as it is known on host mycelium. No typical verticillate conidiophores were present. Usually the sporogenous cells arose singly, directly from the mycelium. Spores were produced on the surface and within the agar medium. The most striking result of this experiment was the fact that the extract of *P. rhodina* supported growth of the parasite nearly equal to that of *P. obtusa*. This simply means that the water soluble internal products of the two species are similar in respect to the materials that favor growth of *C. parasiticum*. The immunity of *P. rhodina* under the conditions tested is not due to the chemical constituents that are easily extracted from the mycelium.

Discussion

The discovery of an undescribed fungus parasitic on other common species of fungi is in itself of sufficient interest to justify a study of the mode of parasitism, host range, and factors affecting growth of the parasite. *Calcarisporium parasiticum* was found to be an excellent test organism for the study of the basic principles of the balanced type of parasitism. It was possible to investigate the stages in the host-parasite relationship, including spore germination, tropism of host hyphae, host range, mode of parasitism, effect on the host, resistance, and growth of the parasite in the absence of the host.

The failure of the parasite spores to germinate in distilled water or on agar medium containing no added natural products is a character common to many parasitic fungi. The necessary spore germination factor may be furnished by a number of natural products and appears to be a common metabolic product of many fungi. It is not destroyed by autoclaving. In these respects *C. parasiticum* seems to be similar to *Piptocephalis virginiana* (Berry and Barnett, 1957), but it is doubtful whether the germination factors required by spores of these two genera are the same.

Growth of germ tubes of a parasite toward host tissue is shown by many host-parasite relationships involving higher plants (Yarwood, 1956). Similar tropism of germ tubes of *Piptocephalis virginiana* toward host hyphae have been described (Berry and Barnett, 1957). There is little evidence that the germ tubes of *C. parasiticum* are attracted to the host hyphae, except within a distance of a few microns of the host. However, the unusual situation in this relationship is that the host hyphae are frequently strongly attracted toward germinating spores of the parasite. The secretion from the germinating spores is effective up to a

distance of about 40 microns, as shown by a change in the growth direction of the host hyphae. It is also of interest that hyphae of *P. rhodina*, which was immune to the parasite in all tests, are attracted to the parasite spores as strongly as are the susceptible hosts.

The mode of parasitism of *C. parasiticum* is believed to be unusual among the fungi, although the details of the process are not understood. No haustoria are produced, as in *Piptocephalis* (Berry and Barnett, 1957; Dobbs and English, 1954), nor is there evidence of toxic secretions that kill the host cells, as shown by *Rhizoctonia solani* (Butler, 1957), *Trichoderma* (Weindling, 1932), or *Papulospora* (Warren, 1948). Contact of the host by the parasite is more nearly like that shown by *Chaetocladium* and *Parasitella simplex* (Burgeff, 1924), but these parasites are said to dissolve the host cell wall at the point of contact. There is no evidence of any dissolving action by *C. parasiticum*. In this respect this mode of parasitism is much like that shown by *Taphrina*, the smuts and certain others, which produce no haustoria and cause no destruction of host cells in the early stages of parasitism. Yet, these parasites quickly establish a compatible nutritional relationship with living host cells.

At the point of contact between *C. parasiticum* and its host the walls are usually flattened and there seems to be some cohesive force holding them together. By use of the oil immersion objective of the microscope it was possible to see a very small cell that is formed at the tip of the parasite hypha where it contacts the host. It has not been determined whether this cell is always present at the points of contact. The buffer cell has not been observed in contacts of *P. rhodina* and the parasite. However, its presence is so frequent that it may be concluded that it has a specific function in the successful act of parasitism. Its function is unknown, but the most attractive theory, based on present knowledge, is that this small cell is a manufacturing unit producing and secreting enzymes or other substances that act to increase the permeability of the plasma membrane of the host at the point of contact, and it possibly then functions to absorb nutrients from the host. There is also some evidence that the presence of the parasite later hastens autolysis of the host. This suggests that secretions from the parasite may pass through the buffer cell and increase the rate of metabolic activity of the host. These ideas have little factual support and they will be difficult to verify. However, it is certain that the act of parasitism involves much more than mere absorption of soluble materials as a sponge absorbs water.

During the greater part of this investigation *C. parasiticum* was believed to belong to the group known as obligate parasites. It was not until an extract of the host mycelium was tested that it was discovered

that the parasite can make continued slow (but not normal) growth in the absence of a living host. Evidence indicates that all of the nutrients necessary for slow abnormal development are present in a glucose-yeast extract medium supplemented with a small amount of autoclaved host extract.

Many other investigators have attempted to culture the so-called "obligate parasites" in the absence of the host and some have enjoyed varying degrees of success. Ayres (1933) succeeded in growing *Dispira cornuta*, a parasite in the Mucorales, on egg yolk and other media rich in organic nitrogen. Hotson and Cutter (1951) obtained growth of *Gymnosporangium juniperi-virginianae* on synthetic media and interpreted the results as nutritional adaptation. *Dothidella nlei* failed to grow on malt extract or potato-dextrose agar but did grow slowly when these media were supplemented with extracts of leaves of *Hevea brasiliensis* or of certain other plants (Blazquez and Owen, 1957). Some of the balanced non-obligate parasites, such as some smuts and *Taphrina*, can be grown without difficulty in pure culture but most of them develop normally and reach full sporulation only in the presence of a living host. This suggests that the host furnishes a favorable environment or required nutrient for the normal development of the parasite.

It was found also that all of the essentials for full normal development of *C. parasiticum* are present within aged mycelium of *P. obtusa* that has been washed and starved in distilled water for as long as 30 days. It is obvious that the parasitic factor is not dependent upon an actively growing mycelium. There is some evidence that the materials absorbed from the host by the parasite are normally utilized by the host, since parasitism reduced the rate of growth.

The fact that the mycelial extract is effective in such small quantities in promoting growth of the parasite suggests that its virtue is not due to a major nutrient. It seems to act more as a growth factor or growth stimulator. The identity of the growth promoting substance is one of the major unsolved questions.

The most popular theories of parasitism have as their basis either the presence in the host of required specific nutrients or the absence in susceptible hosts of inhibitory compounds. It seems doubtful if parasitism by *C. parasiticum* can be explained completely by either of these theories. Mycelial extracts of *Physalospora rhodina*, an immune species, are nearly as effective in promoting growth of the parasite as the extract of *P. obtusa*, a closely related, highly susceptible species. Therefore, the reasons for this difference must be sought at some point before the nutrients in the host reach the parasite. It is possible, of course, that an unstable inhibitor exists in the living mycelium of *P. rhodina* and is

destroyed in the process of extraction, but this seems doubtful. It seems more probable that the parasite is unable to absorb the needed nutrients from *P. rhodina*.

It is concluded that a successful parasitic relationship of *Carcari-sporium parasiticum* is dependent upon (1) intimate contact with a potential host fungus, (2) the presence of specific nutrients in the host mycelium, (3) increased permeability of the host cells, and (4) ability of the parasite to absorb the nutrients from the host. After the parasitic relationship has been established with the host another set of factors may determine the degree of susceptibility or resistance. The higher degree of susceptibility of *Physalospora ilicis* and *Coniothyrium* sp. on a medium containing greater amounts of yeast extract or Casamino Acids is probably due to a difference in materials within the mycelium, possibly the soluble nitrogenous compounds. The known steps leading to parasitic and saprophytic growth are summarized in Figure 47.

The discovery of a mutant that produces a greater amount of mycelium and fewer conidia than the parent culture has made it possible to grow and measure the fungus by diameter of colony and by dry weight of the parasite and to study more easily the physiology of the parasite. This change in the growth habit of the parasite is believed to represent a true mutation, since it appeared several months after the isolate was discovered and after it had been transferred many times. The mutation was accompanied by little loss in its ability to parasitize susceptible hosts.

It is interesting that the known host range of *C. parasiticum* is limited to 3 species of *Physalospora*, *Botryosphaeria ribis* (taxonomically closely related to *Physalospora*), and some species of *Dothiorella*, *Diplodia* and *Coniothyrium* that may have *Physalospora* as their perfect stages. It may be concluded that these fungi have physiological similarities. If the host range studies can be extended to authentic cultures of all known species of *Physalospora* and to many species of closely related genera, as well as many other pycnidium-forming fungi, it may be possible to use susceptibility under standard conditions as a taxonomic character to verify identification or tentatively identify species within this group of fungi. This would be particularly valuable in grouping non-sporulating cultures isolated from die-backs of trees and shrubs.

The present study has uncovered many more problems of parasitism than have been solved. It is proposed that the more important of these problems be investigated separately and more intensively. The problem of identifying the spore germination factor, the substance causing the tropism of host hyphae toward the parasite, and the growth stimulator in the host extract is one which may require much time and the combined efforts of the mycologists and biochemists using modern methods.

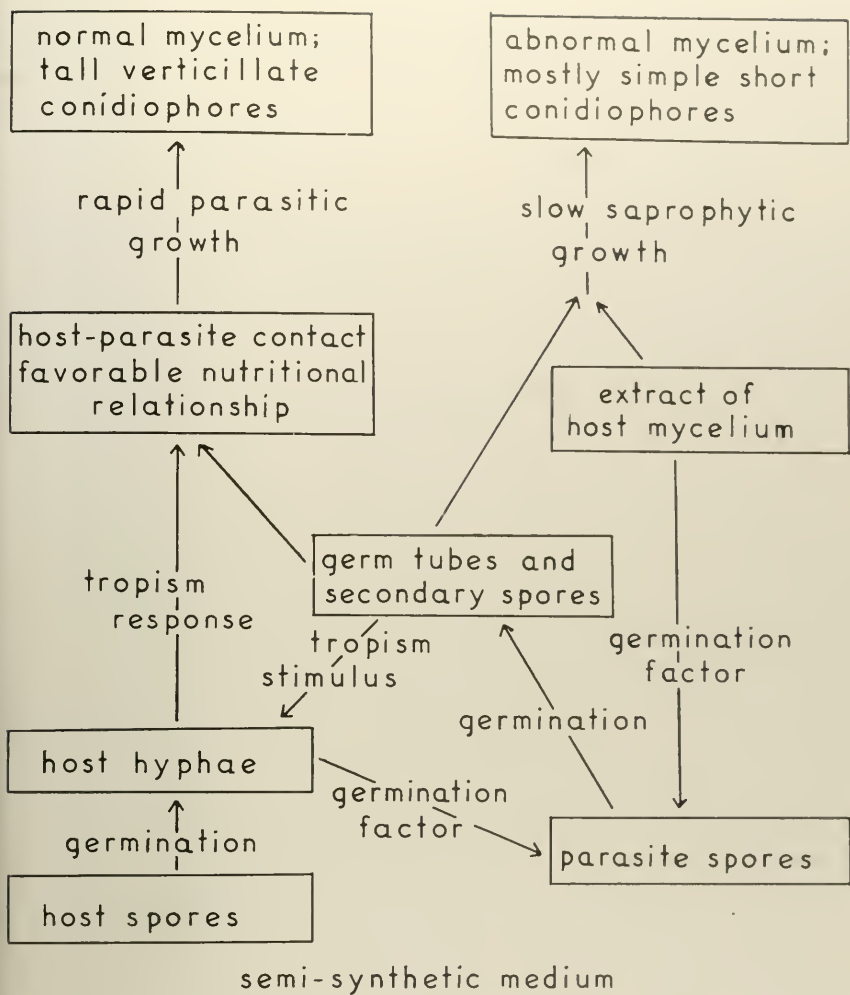


Figure 47. *Calcarisporium parasiticum*. Diagram presenting a summary of the steps leading to parasitic growth (left side of page) and saprophytic growth (right side of page).

of detection. The problem of increasing host resistance by changing its nutrients is one of much interest to plant pathologists, and a study of factors affecting growth of *C. parasiticum* in the absence of a living host should attract the interest of the fungus physiologist.

Summary

Calcarisporium parasiticum was found to parasitize the following species: *Phylospora obtusa*, *P. glandicola* (*Dothovella quercina*), *P.*

ilicis, *Botryosphaeria ribis*, *Guignardia bidwellii*, *diplodia pinea*, *Dothiorrella* sp. and *Coniothyrium* sp. Six species of *Physalospora* and more than 40 other fungi tested were not parasitized. Differences between isolates of the same species were apparent. Four isolates of the parasite were nearly equal in virulence.

The degree of susceptibility of *P. ilicis* and *Coniothyrium* sp. was increased by growth on a medium high in concentration of amino acids. A medium with high sugar concentration increased resistance. *P. rhodina* remained immune under all conditions tested.

The spores of *C. parasiticum* failed to germinate in distilled water or on water agar. The addition of natural products from various sources or from a living host mycelium resulted in a high percentage of germination. Germination is usually followed quickly by the production of secondary spores but further development of the parasite occurred only in the presence of a living host or upon the addition of an extract of the mycelium of certain fungi. The mycelial extract permitted slow continued abnormal development, but normal development occurred only on a living host. Extract of *P. rhodina* was nearly as effective in promoting growth of the parasite as was that of *P. obtusa*. The active principle in the extract is stable during autoclaving.

The germinating spores of *C. parasiticum* secrete a substance that results in a positive tropism of the host hyphae. Firm contact is established between host and parasite but there is no penetration of the host cells. At the tip of the parasite hypha that contacts the host there is formed a small cell that is believed to function in some way to establish a compatible nutritional relationship with the host. Development of the parasite on the host is rapid and sporulation is abundant. A brief description of the morphology of the parasite, with illustrations, is given.

The only visible harmful effect of the parasite on the host is in the reduction in growth rate and possibly an increase in the rate of autolysis. The dry weights of the host-parasite cultures were less than that of the host alone under the same conditions. The carbon source, nitrogen source and pH of the host medium had little or no effect on the parasite growing on *Physalospora obtusa*.

The usual appearance of *C. parasiticum* on glucose-yeast extract agar supplemented with host extract is somewhat yeast-like. Spores are produced on the surface and within the agar, but relatively little mycelium is produced. In one of these cultures there developed a mycelial mutant characterized by much more mycelium and fewer spores than the parent. This mutant was parasitic and only slightly less virulent than the parent culture. A relatively high concentration of amino acid in an agar medium supplemented with host extract favored the growth of both the mutant and its parent culture in the absence of a living host.

Calcarisporium parasiticum is an excellent test fungus for more intensive studies on the balanced type of parasitism.

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